PCT/JP2004/010442
12.11.2004

PA 1222869

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APPLICATION NUMBER: *60/488,924*
FILING DATE: *July 21, 2003*

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Express Mail Label No.: EV 138525155US
Date of Deposit: July 21, 2003

Attorney Docket No. 25371-022

60/488724 07/21/03

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c)

INVENTORS/APPLICANTS

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TITLE OF THE INVENTION

METHOD FOR DIAGNOSING COLORECTAL CANCERS

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ENCLOSED APPLICATION PARTS

☒ **Specification** — Number of Pages: 43
☒ **Sequence Listing** — Number of Pages: 9
☒ **Drawings** (☐ Formal; ☒ Informal) — Number of Sheets: 4 pages; (Figures 1-4)
☐ **Other (Please Specify)** — Number of Pages:

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government:

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are:

METHOD OF PAYMENT

☒ A check in the amount of $160.00 is enclosed to cover the filing fees of the Provisional application.
☐ The Commissioner is hereby authorized to charge $160.00 is enclosed to cover the filing fees of the Provisional application to Deposit Account No. 50-0311, Reference No_______.
☒ The Commissioner is hereby authorized to charge additional fees or credit any overpayment to Deposit Account No. 50-0311, Reference No. 25371-022.

Respectfully submitted,

Date: July 21, 2003

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TRA 1816270v1

Express Mail Label No.: EV 138525155US
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METHOD FOR DIAGNOSING COLORECTAL CANCERS

FIELD OF THE INVENTION

The invention relates to methods of diagnosing colorectal cancers.

BACKGROUND OF THE INVENTION

Colorectal cancer (CRC) is one of the most common solid tumors worldwide. In 2000 nearly 940,000 individuals were diagnosed with colon cancer and approximately 579,000 died from it (1,2). Although great progress has been made in recent years with regard to diagnosis and treatment, the prognosis for patients with advanced colon cancers remains poor. Hence, discovery of a sensitive and specific diagnostic biomarker for detection of early-stage carcinomas, and development of more effective but less harmful therapeutic drugs, are matters of pressing concern. Furthermore, effective preventive strategies would release many people from fear of this life-threatening disease. To achieve those goals, the detailed molecular mechanisms underlying colorectal carcinogenesis must first be well understood.

Recent molecular studies have revealed that colorectal carcinogenesis involves an accumulation of genetic alterations within a cell lineage, which include not only mutations that inactivate tumor suppressor genes and activate proto-oncogenes, but also amplifications of DNA and/or losses of DNA in certain chromosomal regions. In addition to those types of changes, epigenetic events such as methylation, loss of imprinting, and/or dysregulated expression resulting from genetic changes or unknown mechanisms underlie the genesis of colorectal tumors.

Genes in the Wnt/wingless signaling pathway play critical roles in differentiation and morphogenesis during embryogenesis. Impaired regulation of this pathway often is a feature of tumors arising in the colon, liver, prostate, stomach, brain, endometrium, or elsewhere (3). One of the key mediators of the pathway is ß-catenin, which plays a pivotal role in cell-to-cell adhesion and signal transduction. In the absence of Wnt signaling, ß-catenin is phosphorylated by a multi-molecular complex composed of ß-catenin, APC protein, Axin1, Axil/conductin (AXIN2), and glycogen synthase kinase 3ß (GSK3ß). ß-catenin is normally down-regulated through ubiquitination and subsequent degradation in the proteosome, but wnt signaling allows ß-catenin to accumulate in the cytoplasm and/or nucleus due to inhibition of GSK3ß. Abnormal intracellular accumulation of ß-catenin as a consequence of genetic alterations in *APC*, *AXIN1*, *AXIN2*, or ß-catenin (*CTNNB1*) genes has been observed in various human cancers

including colorectal and hepatocellular carcinomas (4). Accumulated ß-catenin forms a complex with the Tcf/LEF transcription factor and up-regulates downstream target genes such as *c-myc* (5) and *cyclinD1* (6, 7). Activation of one or more of these genes can contribute to processes that confer malignant properties on colon-carcinoma cells.

The family of fibroblast growth factors (FGFs) comprises a group of 23 secreted polypeptides, which mediate their signals upon binding with one or two of five types of cognate receptors. FGFs play important roles in embryonic development, cell growth, morphogenesis, tissue repair, inflammation, and angiogenesis (8). For example, FGF4, FGF8, FGF10, FGF18 and FGF20 are involved in limb development (9, 10); FGF8 participates in the signaling cascade in the organogenesis of midbrain-hindbrain (11); and FGF10 appears to be essential for development of the lung. Wnt signals control some of the FGFs involved in limb initiation and tooth organogenesis (12, 13). Apart from a crucial role in organogenesis, FGF2 stimulates tissue repair in the adult (14, 15). However, inappropriate expression of FGFs and/or their receptors occurs in a wide range of human tumors including bladder, cervical and gastric cancers (16, 17). Among members of the FGF family, FGF18 is the one that most closely resembles FGF8; and, like FGF2, FGF18 stimulates proliferation of NIH3T3 cells (18), osteoblasts (19), chondrocytes (19), and glial cells (20), and induces neurite outgrowth of PC12 rat pheochromocytoma cells (21).

cDNA microarray technologies have enabled to obtain comprehensive profiles of gene expression in normal and malignant cells, and compare the gene expression in malignant and corresponding normal cells (Okabe et al., Cancer Res 61:2129-37 (2001); Kitahara et al., Cancer Res 61: 3544-9 (2001); Lin et al., Oncogene 21:4120-8 (2002); Hasegawa et al., Cancer Res 62:7012-7 (2002)). This approach enables to disclose the complex nature of cancer cells, and helps to understand the mechanism of carcinogenesis. Identification of genes that are deregulated in tumors can lead to more precise and accurate diagnosis of individual cancers, and to develop novel therapeutic targets (Bienz and Clevers, Cell 103:311-20 (2000)). To disclose mechanisms underlying tumors from a genome-wide point of view, and discover target molecules for diagnosis and development of novel therapeutic drugs, the present inventors have been analyzing the expression profiles of tumor cells using a cDNA microarray of 23040 genes (Okabe et al., Cancer Res 61:2129-37 (2001); Kitahara et al., Cancer Res 61:3544-9 (2001); Lin et al., Oncogene 21:4120-8 (2002); Hasegawa et al., Cancer Res 62:7012-7 (2002)).

Studies designed to reveal mechanisms of carcinogenesis have already facilitated identification of molecular targets for anti-tumor agents. For example, inhibitors of

farnexyltransferase (FTIs) which were originally developed to inhibit the growth-signaling pathway related to Ras, whose activation depends on posttranslational farnesylation, has been effective in treating Ras-dependent tumors in animal models (He et al., Cell 99:335-45 (1999)). Clinical trials on human using a combination or anti-cancer drugs and anti-HER2 monoclonal antibody, trastuzumab, have been conducted to antagonize the proto-oncogene receptor HER2/neu; and have been achieving improved clinical response and overall survival of breast-cancer patients (Lin et al., Cancer Res 61:6345-9 (2001)). A tyrosine kinase inhibitor, STI-571, which selectively inactivates bcr-abl fusion proteins, has been developed to treat chronic myelogenous leukemias wherein constitutive activation of bcr-abl tyrosine kinase plays a crucial role in the transformation of leukocytes. Agents of these kinds are designed to suppress oncogenic activity of specific gene products (Fujita et al., Cancer Res 61:7722-6 (2001)). Therefore, gene products commonly up-regulated in cancerous cells may serve as potential targets for developing novel anti-cancer agents.

It has been demonstrated that CD8+ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumor-associated antigens (TAAs) presented on MHC Class I molecule, and lyse tumor cells. Since the discovery of MAGE family as the first example of TAAs, many other TAAs have been discovered using immunological approaches (Boon, Int J Cancer 54: 177-80 (1993); Boon and van der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J Exp Med 178: 489-95 (1993); Kawakami et al., J Exp Med 180: 347-52 (1994)). Some of the discovered TAAs are now in the stage of clinical development as targets of immunotherapy. TAAs discovered so far include MAGE (van der Bruggen et al., Science 254: 1643-7 (1991)), gp100 (Kawakami et al., J Exp Med 180: 347-52 (1994)), SART (Shichijo et al., J Exp Med 187: 277-88 (1998)), and NY-ESO-1 (Chen et al., Proc Natl Acad Sci USA 94: 1914-8 (1997)). On the other hand, gene products which had been demonstrated to be specifically overexpressed in tumor cells, have been shown to be recognized as targets inducing cellular immune responses. Such gene products include p53 (Umano et al., Brit J Cancer 84: 1052-7 (2001)), HER2/neu (Tanaka et al., Brit J Cancer 84: 94-9 (2001)), CEA (Nukaya et al., Int J Cancer 80: 92-7 (1999)), and so on.

In spite of significant progress in basic and clinical research concerning TAAs (Rosenbeg et al., Nature Med 4: 321-7 (1998); Mukherji et al., Proc Natl Acad Sci USA 92: 8078-82 (1995); Hu et al., Cancer Res 56: 2479-83 (1996)), only limited number of candidate TAAs for the treatment of adenocarcinomas, including colorectal cancer, are available. TAAs abundantly expressed in cancer cells, and at the same time which expression is restricted to cancer cells

would be promising candidates as immunotherapeutic targets. Further, identification of new TAAs inducing potent and specific antitumor immune responses is expected to encourage clinical use of peptide vaccination strategy in various types of cancer (Boon and can der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J Exp Med 178: 489-95 (1993); Kawakami et al.,J Exp Med 180: 347-52 (1994); Shichijo et al., J Exp Med 187: 277-88 (1998); Chen et al., Proc Natl Acad Sci USA 94: 1914-8 (1997); Harris, J Natl Cancer Inst 88: 1442-5 (1996); Butterfield et al., Cancer Res 59: 3134-42 (1999); Vissers et al., Cancer Res 59: 5554-9 (1999); van der Burg et al., J Immunol 156: 3308-14 (1996); Tanaka et al., Cancer Res 57: 4465-8 (1997); Fujie et al., Int J Cancer 80: 169-72 (1999); Kikuchi et al., Int J Cancer 81: 459-66 (1999); Oiso et al., Int J Cancer 81: 387-94 (1999)).

It has been repeatedly reported that peptide-stimulated peripheral blood mononuclear cells (PBMCs) from certain healthy donors produce significant levels of IFN-γ in response to the peptide, but rarely exert cytotoxicity against tumor cells in an HLA-A24 or –A0201 restricted manner in ^{51}Cr-release assays (Kawano et al., Cance Res 60: 3550-8 (2000); Nishizaka et al., Cancer Res 60: 4830-7 (2000); Tamura et al., Jpn J Cancer Res 92: 762-7 (2001)). However, both of HLA-A24 and HLA-A0201 are one of the popular HLA alleles in Japanese, as well as Caucasian (Date et al., Tissue Antigens 47: 93-101 (1996); Kondo et al., J Immunol 155: 4307-12 (1995); Kubo et al., J Immunol 152: 3913-24 (1994); Imanishi et al., Proceeding of the eleventh International Hictocompatibility Workshop and Conference Oxford University Press, Oxford, 1065 (1992); Williams et al., Tissue Antigen 49: 129 (1997)). Thus, antigenic peptides of carcinomas presented by these HLAs may be especially useful for the treatment of carcinomas among Japanese and Caucasian. Further, it is known that the induction of low-affinity CTL in vitro usually results from the use of peptide at a high concentration, generating a high level of specific peptide/MHC complexes on antigen presenting cells (APCs), which will effectively activate these CTL (Alexander-Miller et al., Proc Natl Acad Sci USA 93: 4102-7 (1996)).

SUMMARY OF THE INVENTION

To search for potential molecular targets for development of novel anti-cancer drugs, we have been analyzing expression profiles of clinical samples from cancer patients using a genome-wide cDNA microarray. In experiments with colon-cancer cells, the gene encoding fibroblast growth factor 18 (*FGF18*) was among those that showed elevated expression. The promoter region of this gene was found to contain putative Tcf4-binding motifs; moreover a

reporter-gene assay using the luciferase activity as a marker, as well as an electromobility-shift assay, indicated that *FGF18* is a downstream transcription target in the ß-catenin/Tcf4 pathway. We showed that exogenous FGF18 promoted growth of NIH3T3 cells in an autocrine manner, and that transfection of *FGF18* siRNAs suppressed growth of colon-cancer cells in culture. Our results indicate that FGF18 is activated in colon cancers as a direct downstream target of the Wnt signaling pathway, and that it might represent a marker for early diagnosis and a molecular target for treatment of CRC.

The invention is based on the discovery of a pattern of gene expression of FGF18 correlated with colorectal cancer (CRC).

Accordingly, the invention features a method of diagnosing or determining a predisposition to CRC in a subject by determining an expression level of FGF18 in a patient derived biological sample, such as tissue sample. A normal cell is one obtained from colorectal tissue. An increase of the level of expression of the FGF18 compared to a normal control level of the gene indicates that the subject suffers from or is at risk of developing CRC.

By normal control level is meant a level of gene expression detected in a normal, healthy individual or in a population of individuals known not to be suffering from CRC. A control level is a single expression pattern derived from a single reference population or from a plurality of expression patterns. For example, the control level can be a database of expression patterns from previously tested cells. A normal individual is one with no clinical symptoms of CRC and no family history of CRC.

An increase in the level of expression of FGF18 detected in a test sample compared to a normal control level indicates the subject (from which the sample was obtained) suffers from or is at risk of developing CRC.

Gene expression is increased 10%, 25%, 50% compared to the control level. Alternately, gene expression is increased 0.1, 0.2, 1, 2, 5, 10 or more fold compared to the control level. Expression is determined by detecting hybridization, *e.g.*, FGF18 gene probe to a gene transcript of the patient-derived tissue sample.

The patient derived tissue sample is any tissue from a test subject, e.g., a patient known to or suspected of having CRC. For example, the tissue contains a colorectal cnacer cell. For example, the tissue is a cell from colon.

The invention further provides methods of identifing an agent that inhibits the expression or activity of FGF18 by contacting a test cell expressing FGF18 with a test agent and determining the expression level or activity of the FGF18. The test cell is a colon cell such as a

colon cell from a colorectal cancer. A decrease of the level compared to a normal control level of the gene indicates that the test agent is an inhibitor of the FGF18 and reduces a symptom of CRC.

The invention also provides a kit with a detection reagent which binds to FGF18 nucleic acid sequences or which binds to a gene product encoded by the nucleic acid sequences.

Therapeutic methods include a method of treating or preventing CRC in a subject by administering to the subject an antisense composition. The antisense composition reduces the expression of a specific target gene, e.g., the antisense composition contains a nucleotide, which is complementary to a nucleic acid sequence of FGF18. Another method includes the steps of administering to a subject an small interfering RNA (siRNA) composition. The siRNA composition reduces the expression of a nucleic acid of FGF18. In yet another method, treatment or prevention of CRC in a subject is carried out by administering to a subject a ribozyme composition. The nucleic acid-specific ribozyme composition reduces the expression of a nucleic acid of FGF18.Suitable mechanisms for in vivo expression of a gene of interest are known in the art.

The invention also includes vaccines and vaccination methods. For example, a method of treating or preventing CRC in a subject is carried out by administering to the subject a vaccine containing a polypeptide encoded by a nucleic acid of FGF18 or an immunologically active fragment such a polypeptide. An immunologically active fragment is a polypeptide that is shorter in length than the full-length naturally-occurring protein and which induces an immune response. For example, an immunologically active fragment at least 8 residues in length and stimulates an immune cell such as a T cell or a B cell. Immune cell stimulation is measured by detecting cell proliferation, elaboration of cytokines (e.g., IL-2), or production of an antibody.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

One advantage of the methods described herein is that the disease is identified prior to detection of overt clinical symptoms. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1A and 1B depict elevated expression of FGF18 in CRCs. Fig. 1A dipicts semi-quantitative RT-PCR analysis of *FGF18* in 12 colon-cancer tissues (T) and their corresponding normal mucosae (N). Expression of *GAPDH* served as an internal control. Fig. 1B depicts immunohistochemical staining of FGF18 in cancerous colon tissues (*a, c*) and corresponding non-cancerous mucosae (*b, d*). (Bars, 0.1 mm)

Fig. 2A and 2B depict a schematic representation of reporter plasmids of FGF18 (A) and result of reporter assay (B). Fig. 2A depicts schematic representation of various reporter plasmids of FGF18. The putative Tcf4-binding motifs are located between -1631 bp and -1625 bp (TBM1), between -1348 bp and -1342 bp (TBM2), and between -190 bp and -184 bp (TBM3) from the transcription-initiation site (TIS). Constructs P1, P2 and P3 contain wild-type binding elements; P2mt or P3mt have 2-bp substitutions in TBM2 or TBM3 respectively. Fig. 2B depicts dual luciferase reporter assay in SW480 cells (*T-bars*, SD; asterisks, Scheffe's *F* test, $p<0.0001$).

Fig. 3 depicts the result of EMSA of the ß-catenin/Tcf4 complex using TBM3-oligonucleotide as a probe. A super-shifted band was observed after addition of anti-ß-catenin antibody (*lanes 2 and 7*) but was not elicited by anti-P53-antibody (*lane 3*). Bands corresponding to the DNA-protein complex were reduced by addition of non-labeled wild-type probe (*lanes 4, 5*), but not by non-labeled mutant probe (*lanes 6, 7*).

Fig. 4. depicts the growth promoting effect of FGF18 (A and B) or the growth suppressive effect of FGF18-siRNAs (C, D, and E). Fig. 4A depicts immunoblotting of Flag-tagged FGF18 protein secreted into culture media. Proteins in the medium and cell lysate were immunoblotted with anti-Flag antibody. NIH3T3 cells were transfected with either pFlagCMV or pFlagCMV-FGF18. Black and open triangles indicate the cellular or secreted forms respectively of tagged FGF18 protein. Fig. 4B depicts microscopic appearance of NIH3T3 cells after incubation in DMEM containing 0.5% FBS (*left*), in the conditioned medium after

transfection with pFlagCMV-FGF18 (*center*), and in the conditioned medium after transfection with pFlagCMV (*right*). Fig. 4C depicts effect of FGF18 siRNAs on expression of FGF18. Semi-quantitative RT-PCR was carried out with RNAs from cells transfected with siRNA-expressing or control plasmids. Giemsa's staining (Fig. 4D)or MTT assay (Fig. 4E) of viable HCT116 cells in response to EGFP-siRNA or FGF18-siRNA. MTT assays were carried out in triplicate (*T-bars*, SD; asterisk, Scheffe's F test, p=0.02).

DETAILED DESCRIPTION

The present invention is based in part on the discovery of elevated expression of FGF18 in cells from colon of patients with CRC. The elevated gene expression was identified by using a comprehensive cDNA microarray system.

Using a cDNA microarray containing 23,040 genes, comprehensive gene-expression profiles of 20 patients were constructed previously. *FGF18* is expressed at high level in CRC patients. In the process candidate molecular marker was selected with the potential of detecting cancer-related proteins in serum or sputum of patients, and some potential targets for development of signal-suppressing strategies in human colorectal cancer were discovered.

FGF18 identified herein are used for diagnostic purposes as marker of CRC and as gene target, the expression of which is altered to treat or alleviate a symptom of CRC.

Unless indicated otherwise, "CRC " is meant to refer to any of the sequences disclosed herein.

By measuring expression of FGF18 in a sample of cells, CRC is diagnosed. Similarly, by measuring the expression of FGF18 in response to various agents, and agents for treating CRC can be identified.

The invention involves determining (*e.g.*, measuring) the expression of FGF18. Using sequence information provided by the GeneBank™ database entries for FGF18 sequence, *FGF18* is detected and measured using techniques well known to one of ordinary skill in the art. For example, sequence within the sequence database entries corresponding to *FGF18*, is used to construct probes for detecting FGF18 RNA sequence in, *e.g.*, northern blot hybridization

analyses. As another example, the sequences can be used to construct primers for specifically amplifying FGF18 in, *e.g*, amplification-based detection methods such as reverse-transcription based polymerase chain reaction.

Expression level of FGF18 in the test cell population, *e.g.*, a patient derived tissues sample is then compared to expression level of the FGF18 in a reference population. The reference cell population includes one or more cells for which the compared parameter is known, *i.e.*, CRC cells or non-CRC cells.

Whether or not a pattern of gene expression in the test cell population compared to the reference cell population indicates CRC or a predisposition thereto depends upon the composition of the reference cell population. For example, if the reference cell population is composed of non-CRC cells, a similar gene expression pattern in the test cell population and reference cell population indicates the test cell population is non-CRC. Conversely, if the reference cell population is made up of CRC cells, a similar gene expression profile between the test cell population and the reference cell population indicates that the test cell population includes CRC cells.

A level of expression of a CRC marker gene in a test cell population is considered altered in levels of expression if its expression level varies from the reference cell population by more than 1.0, 1.5, 2.0, 5.0, 10.0 or more fold from the expression level of the corresponding *FGF18* in the reference cell population.

Differential gene expression between a test cell population and a reference cell population is normalized to a control nucleic acid, *e.g.* a housekeeping gene. For example, a control nucleic acid is one which is known not to differ depending on the endometriotic or non-endometriotic state of the cell. Expression levels of the control nucleic acid in the test and reference nucleic acid can be used to normalize signal levels in the compared populations. Control genes include ß-actin, glyceraldehyde 3- phosphate dehydrogenase or ribosomal protein P1.

The test cell population is compared to multiple reference cell populations. Each of the multiple reference populations may differ in the known parameter. Thus, a test cell population may be compared to a second reference cell population known to contain, *e.g.*, CRC cells, as well as a second reference population known to contain, *e.g.*, non-CRC cells (normal cells).

The test cell is included in a tissue type or cell sample from a subject known to contain, or to be suspected of containing, CRC cells.

The test cell is obtained from a bodily tissue or a bodily fluid, *e.g.*, biological fluid (such as blood or urine). For example, the test cell is purified from a tissue. Preferably, the test cell population comprises an epithelial cell. The epithelial cell is from tissue known to be or suspected to be a CRC.

Cells in the reference cell population are derived from a tissue type as similar to test cell. Optionally, the reference cell population is a cell line, *e.g.* a CRC cell line (positive control) or a normal non-CRC cell line (negative control). Alternatively, the control cell population is derived from a database of molecular information derived from cells for which the assayed parameter or condition is known.

The subject is preferably a mammal. The mammal can be, *e.g.*, a human, non-human primate, mouse, rat, dog, cat, horse, or cow.

Expression of FGF18 disclosed herein is determined at the protein or nucleic acid level using methods known in the art. For example, Northern hybridization analysis using probes which specifically recognize the sequence can be used to determine gene expression. Alternatively, expression is measured using reverse-transcription-based PCR assays, *e.g.*, using primers specific for *FGF18*. Expression is also determined at the protein level, *i.e.*, by measuring the levels of polypeptide encoded by the gene product described herein, or biological activity thereof. Such methods are well known in the art and include, *e.g.*, immunoassays based on antibodies to protein encoded by *FGF 18*. The biological activity of the protein encoded by the gene is also well known.

Diagnosing CRC

CRC is diagnosed by measuring the level of expression of FGF18 from a test population of cells, (*i.e.*, a patient derived biological sample). Preferably, the test cell population contains an epithelial cell, e.g., a cell obtained from colon tissue. Gene expression is also measured from blood or other bodily fluids such as urine. Other biological samples can be used for measuring the protein level. For example, the protein level in the blood, or serum derived from subject to be diagnosed can be measured by immunoassay or biological assay.

Expression of FGF18 is determined in the test cell or biological sample and compared to the expression of the normal control level. A normal control level is an expression profile of FGF18 typically found in a population known not to be suffering from CRC. An increase of

the level of expression in the patient derived tissue sample of FGF18 indicates that the subject is suffering from or is at risk of developing CRC.

When *FGF18* is altered in the test population compared to the normal control level indicates that the subject suffers from or is at risk of developing CRC.

Identifying Agents that inhibit FGF18 expression or activity

An agent that inhibits the expression or activity of FGF18 is identified by contacting a test cell population expressing FGF18 with a test agent and determining the expression level or activity of FGF18. A decrease of expression or activity in the presence of the agent compared to the normal control level (or compared to the level in the absence of the test agent) indicates the agent is an inhibitor of FGF18 and useful to inhibit CRC.

The test cell population is any cell expressing FGF18. For example, the test cell population contains an epithelial cell, such as a cell is or derived from colon. For example, the test cell is an immortalized cell line derived from colorectal cancer. Alternatively, the test cell is a cell, which has been transfected with *FGF18* or which has been transfected with a regulatory sequence (*e.g.* promoter sequence) from FGF18 operably linked to a reporter gene. Furthermore, a candidate compound that interferes the binding between ß-catenin/Tcf4 binding motif and ß-catenin/Tcf4 complex, may be identified as an inhibiting agent of FGF18.

Assessing efficacy of treatment of CRC *in a subject*

The differentially expressed FGF18 identified herein also allow for the course of treatment of CRC to be monitored. In this method, a test cell population is provided from a subject undergoing treatment for CRC. If desired, test cell populations are obtained from the subject at various time points before, during, or after treatment. Expression of FGF18, in the cell population is then determined and compared to a reference cell population which includes cells whose CRC state is known. The reference cells have not been exposed to the treatment.

If the reference cell population contains no CRC cells, a similarity in expression between FGF18 in the test cell population and the reference cell population indicates that the treatment is efficacious. However, a difference in expression between FGF18 in the test population and a normal control reference cell population indicates the less favorable clinical outcome or prognosis.

By "efficacious" is meant that the treatment leads to a reduction in expression of a pathologically up-regulated gene, increase in expression of a pathologically down-regulated gene

or a decrease in size, prevalence, or metastatic potential of colorectal tumors in a subject. When treatment is applied prophylactically, "efficacious" means that the treatment retards or prevents CRC from forming or retards, prevents, or alleviates a symptom of clinical CRC. Assesment of colorectal tumors are made using standard clinical protocols.

Efficaciousness is determined in association with any known method for diagnosing or treating CRC. CRC is diagnosed for example, by identifying symptomatic anomalies,.

Selecting a therapeutic agent for treating CRC *that is approprlate for a particular individual*

Differences in the genetic makeup of individuals can result in differences in their relative abilities to metabolize various drugs. An agent that is metabolized in a subject to act as an anti-CRC agent can manifest itself by inducing a change in gene expression pattern in the subject's cells from that characteristic of an CRC state to a gene expression pattern characteristic of a non- CRC state. Accordingly, the differentially expressed FGF18 disclosed herein allow for a putative therapeutic or prophylactic inhibitor of CRC to be tested in a test cell population from a selected subject in order to determine if the agent is a suitable inhibitor of CRC in the subject.

To identify an inhibitor of CRC, that is appropriate for a specific subject, a test cell population from the subject is exposed to a therapeutic agent, and the expression of FGF18 is determined.

The test cell population contains a CRC cell expressing FGF18. Preferably, the test cell is an epithelial cell. For example a test cell population is incubated in the presence of a candidate agent and the pattern of gene expression of the test sample is measured and compared to one or more reference profiles, *e.g.*, a CRC reference expression profile or a non-CRC reference expression profile.

A decrease in expression of FGF18 in a test cell population relative to a reference cell population containing CRC is indicative that the agent is therapeutic.

The test agent can be any compound or composition. For example, the test agents are immunomodulatory agents.

Screening assays for identifying therapeutic agents

FGF18 disclosed herein can also be used to identify candidate therapeutic agents for treating a CRC. The method is based on screening a candidate therapeutic agent to determine if

it converts an expression profile of FGF18 characteristic of a CRC state to a pattern indicative of a non-CRC state.

In the method, a cell is exposed to a test agent or a combination of test agents (sequentially or consequentially) and the expression of FGF18 in the cell is measured. The expression level of FGF18 in the test population is compared to expression level of FGF18 in a reference cell population that is not exposed to the test agent.

An agent effective in suppressing expression of over-expressed genes is deemed to lead to a clinical benefit. Such compounds are further tested for the ability to prevent CRC growth.

In a further embodiment, the present invention provides methods for screening candidate agents which are potential targets in the treatment of CRC. As discussed in detail above, by controlling the expression levels or activities of marker gene, one can control the onset and progression of CRC. Thus, candidate agents, which are potential targets in the treatment of CRC, can be identified through screenings that use the expression levels and activities of marker gene as indices. In the context of the present invention, such screening may comprise, for example, the following steps:

a) contacting a test compound with a polypeptide encoded by a nucleic acid of FGF18;
b) detecting the binding activity between the polypeptide and the test compound; and
c) selecting a compound that binds to the polypeptide

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a candidate compound with a cell expressing FGF18, and
b) selecting a compound that reduces the expression level of FGF18.

Cells expressing marker gene include, for example, cell lines established from CRC; such cells can be used for the above screening of the present invention.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a test compound with a polypeptide encoded by a nucleic acid of FGF18;
b) detecting the biological activity of the polypeptide of step (a); and
c) selecting a compound that suppresses the biological activity of the polypeptide encoded by a nucleic acid of FGF18 in comparison with the biological activity detected in the absence of the test compound.

A protein required for the screening can be obtained as a recombinant protein using the nucleotide sequence of the marker gene. Based on the information of the marker gene, one

skilled in the art can select any biological activity of the protein as an index for screening and a measurement method based on the selected biological activity. Preferably, cell proliferative activity of FGF18 may be selected as the biological activity. The cell proliferative activity is detected by proliferation of cell line such as NIH3T3.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of FGF18 and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced
b) measuring the activity of said reporter gene; and
c) selecting a compound that reduces the expression level of said reporter gene, as compared to a control.

Suitable reporter genes and host cells are well known in the art. The reporter construct required for the screening can be prepared by using the transcriptional regulatory region of a marker gene. When the transcriptional regulatory region of a marker gene has been known to those skilled in the art, a reporter construct can be prepared by using the previous sequence information. A DNA comprising ß-catenin/Tcf4 binding motif can be used for the transcriptional regulatory region in the present invention. A DNA comprising nucleotide sequence set forth in SEQ ID NO :24 is preferable as the transcriptional regulatory region. When the transcriptional regulatory region of a marker gene remains unidentified, a nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library based on the nucleotide sequence information of the marker gene.

Additionally, the screening method of the present invention may comprise the following steps:

a) contacting a DNA comprising the ß-catenin/Tcf4 binding motief in the transcriptional regulatory region of FGF18 gene with ß-catenin/Tcf4 complex in the presence or absence of candidate compound;
b) ditecting the binding of the DNA and the ß-catenin/Tcf4 complex; and
c) selecting a compound that inhibits the binding of the ß-catenin/Tcf4 complex with the DNA, , as compared to a control.

In the present invention, the binding of the DNA and the ß-catenin/Tcf4 complex can be ditected with mobolity shift assay (EMSA).

The compound isolated by the screening is a candidate for drugs that inhibit the activity

of the protein encoded by marker gene and can be applied to the treatment or prevention of CRC.

Moreover, compound in which a part of the structure of the compound inhibiting the activity of protein encoded by marker gene is converted by addition, deletion and/or replacement are also included in the compounds obtainable by the screening method of the present invention.

When administrating the compound isolated by the method of the invention as a pharmaceutical for humans and other mammals, such as mice, rats, guinea-pigs, rabbits, chicken, cats, dogs, sheep, pigs, cattle, monkeys, baboons, and chimpanzees, the isolated compound can be directly administered or can be formulated into a dosage form using known pharmaceutical preparation methods. For example, according to the need, the drugs can be taken orally, as sugar-coated tablets, capsules, elixirs and microcapsules, or non-orally, in the form of injections of sterile solutions or suspensions with water or any other pharmaceutically acceptable liquid. For example, the compounds can be mixed with pharmaceutically acceptable carriers or media, specifically, sterilized water, physiological saline, plant-oils, emulsifiers, suspending agents, surfactants, stabilizers, flavoring agents, excipients, vehicles, preservatives, binders, and such, in a unit dose form required for generally accepted drug implementation. The amount of active ingredients in these preparations makes a suitable dosage within the indicated range acquirable.

Examples of additives that can be mixed to tablets and capsules are, binders such as gelatin, corn starch, tragacanth gum and arabic gum; excipients such as crystalline cellulose; swelling agents such as corn starch, gelatin and alginic acid; lubricants such as magnesium stearate; sweeteners such as sucrose, lactose or saccharin; and flavoring agents such as peppermint, Gaultheria adenothrix oil and cherry. When the unit-dose form is a capsule, a liquid carrier, such as an oil, can also be further included in the above ingredients. Sterile composites for injections can be formulated following normal drug implementations using vehicles such as distilled water used for injections.

Physiological saline, glucose, and other isotonic liquids including adjuvants, such as D-sorbitol, D-mannnose, D-mannitol, and sodium chloride, can be used as aqueous solutions for injections. These can be used in conjunction with suitable solubilizers, such as alcohol, specifically ethanol, polyalcohols such as propylene glycol and polyethylene glycol, non-ionic surfactants, such as Polysorbate 80 (TM) and HCO-50.

Sesame oil or Soy-bean oil can be used as a oleaginous liquid and may be used in conjunction with benzyl benzoate or benzyl alcohol as a solubilizer and may be formulated with a buffer, such as phosphate buffer and sodium acetate buffer; a pain-killer, such as procaine hydrochloride; a stabilizer, such as benzyl alcohol and phenol; and an anti-oxidant. The

prepared injection may be filled into a suitable ampule.

Methods well known to one skilled in the art may be used to administer the pharmaceutical composition of the present inevntion to patients, for example as intraarterial, intravenous, or percutaneous injections and also as intranasal, transbronchial, intramuscular or oral administrations. The dosage and method of administration vary according to the body-weight and age of a patient and the administration method; however, one skilled in the art can routinely select a suitable metod of administration. If said compound is encodable by a DNA, the DNA can be inserted into a vector for gene therapy and the vector administered to a patient to perform the therapy. The dosage and method of administration vary according to the body-weight, age, and symptoms of the patient but one skilled in the art can suitably select them.

For example, although the dose of a compound that binds to the protein of the present invention and regulates its activity depends on the symptoms, the dose is about 0.1 mg to about 100 mg per day, preferably about 1.0 mg to about 50 mg per day and more preferably about 1.0 mg to about 20 mg per day, when administered orally to a normal adult (weight 60 kg).

When administering parenterally, in the form of an injection to a normal adult (weight 60 kg), although there are some differences according to the patient, target organ, symptoms and method of administration, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg per day, preferably about 0.1 to about 20 mg per day and more preferably about 0.1 to about 10 mg per day. Also, in the case of other animals too, it is possible to administer an amount converted to 60 kgs of body-weight.

Assessing the prognosis of a subject with CRC

Also provided is a method of assessing the prognosis of a subject with CRC by comparing the expression of FGF18 in a test cell population to the expression of the gene in a reference cell population derived from patients over a spectrum of disease stages. By comparing gene expression of FGF18 in the test cell population and the reference cell population(s), or by comparing the pattern of gene expression over time in test cell populations derived from the subject, the prognosis of the subject can be assessed.

An increase of expression of FGF18 compared to a normal control indicates less favorable prognosis. A decrease in expression of FGF18 indicates a more favorable prognosis for the subject.

Kits

The invention also includes a CRC-detection reagent, e.g., a nucleic acid that specifically binds to or identifies FGF18 nucleic acids such as oligonucleotide sequences, which are complementary to a portion of a FGF18 nucleic acid or antibodies which bind to proteins encoded by a FGF18 nucleic acid. The reagents are packaged together in the form of a kit. The reagents are packaged in separate containers, e.g., a nucleic acid or antibody (either bound to a solid matrix or packaged separately with reagents for binding them to the matrix), a control reagent (positive and/or negative), and/or a detectable label. Instructions (e.g., written, tape, VCR, CD-ROM, etc.) for carrying out the assay are included in the kit. The assay format of the kit is a Northern hybridization or a sandwich ELISA known in the art.

For example, CRC detection reagent is immobilized on a solid matrix such as a porous strip to form at least one CRC detection site. The measurement or detection region of the porous strip may include a plurality of sites containing a nucleic acid. A test strip may also contain sites for negative and/or positive controls. Alternatively, control sites are located on a separate strip from the test strip. Optionally, the different detection sites may contain different amounts of immobilized nucleic acids, *i.e.*, a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test sample, the number of sites displaying a detectable signal provides a quantitative indication of the amount of CRC present in the sample. The detection sites may be configured in any suitably detectable shape and are typically in the shape of a bar or dot spanning the width of a teststrip.

Methods of inhibiting CRC

The invention provides a method for treating or alleviating a symptom of CRC in a subject by decreasing expression or activity of FGF18. Therapeutic compounds are administered prophylactically or therapeutically to subject suffering from (or susceptible to) developing CRC. Administration can be systemic or local. Such subjects are identified using standard clinical methods or by detecting an aberrant level of expression or activity of FGF18. Therapeutic agents include inhibitors of cell proliferation.

The method includes decreasing the expression, or function, or both, of gene products of FGF18 Expression is inhibited in any of several ways known in the art. For example, expression is inhibited by administering to the subject a nucleic acid that inhibits, or antagonizes,

the expression of the over-expressed gene, e.g., an antisense oligonucleotide or small interfering RNA which disrupts expression of the over-expressed gene.

As noted above, antisense nucleic acids corresponding to the nucleotide sequence of FGF18 can be used to reduce the expression level of the FGF18. Antisense nucleic acids corresponding to the nucleotide sequence of FGF18 that are up-regulated in CRC are useful for the treatment of CRC. Specifically, the antisense nucleic acids of the present invention may act by binding to the nucleotide sequence of FGF18 or mRNA corresponding thereto, thereby inhibiting the transcription or translation of the gene, promoting the degradation of the mRNA, and/or inhibiting the expression of protein encoded by a nucleic acid of FGF18, finally inhibiting the function of the proteins . The term "antisense nucleic acids" as used herein encompasses both nucleotides that are entirely complementary to the target sequence and those having a mismatch of nucleotide, so long as the antisense nucleic acids can specifically hybridize to the target sequences. For example, the antisense nucleic acids of the present invention include polynucleotides that have a homology of at least 70% or higher, preferably at 80% or higher, more preferably 90% or higher, even more preferably 95% or higher over a span of at least 15 continuous nucleotides. Algorithms known in the art can be used to determine the homology.

The antisense nucleic acid derivatives of the present invention act on cells producing the protein encoded by marker gene by binding to the DNA or mRNA encoding the protein, inhibiting their transcription or translation, promoting the degradation of the mRNA, and inhibiting the expression of the protein, thereby resulting in the inhibition of the protein function.

An antisense nucleic acid derivative of the present invention can be made into an external preparation, such as a liniment or a poultice, by mixing with a suitable base material which is inactive against the derivative.

Also, as needed, the derivatives can be formulated into tablets, powders, granules, capsules, liposome capsules, injections, solutions, nose-drops and freeze-drying agents by adding excipients, isotonic agents, solubilizers, stabilizers, preservatives, pain-killers, and such. These can be prepared by following known methods.

The antisense nucleic acids derivative is given to the patient by directly applying onto the ailing site or by injecting into a blood vessel so that it will reach the site of ailment. An antisense-mounting medium can also be used to increase durability and membrane-permeability. Examples are, liposomes, poly-L-lysine, lipids, cholesterol, lipofectin or derivatives of these.

The dosage of the antisense nucleic acid derivative of the present invention can be

adjusted suitably according to the patient's condition and used in desired amounts. For example, a dose range of 0.1 to 100 mg/kg, preferably 0.1 to 50 mg/kg can be administered.

The antisense nucleic acids of the invention inhibit the expression of the protein of the invention and is thereby useful for suppressing the biological activity of a protein of the invention. Also, expression-inhibitors, comprising the antisense nucleic acids of the invention, are useful since they can inhibit the biological activity of a protein of the invention.

The antisense nucleic acids of present invention include modified oligonucleotides. For example, thioated nucleotides may be used to confer nuclease resistance to an oligonucleotide.

Also, a siRNA against marker gene can be used to reduce the expression level of the marker gene. By the term "siRNA" is meant a double stranded RNA molecule which prevents translation of a target mRNA. Standard techniques of introducing siRNA into the cell are used, including those in which DNA is a template from which RNA is transcribed. In the context of the present invention, the siRNA comprises a sense nucleic acid sequence and an anti-sense nucleic acid sequence against an upregulated marker gene, such as *FGF18*. The siRNA is constructed such that a single transcript has both the sense and complementary antisense sequences from the target gene, e.g., a hairpin.

The method is used to alter the expression in a cell of an upregulated, e.g., as a result of malignant transformation of the cells. Binding of the siRNA to a transcript corresponding to FGF18 in the target cell results in a reduction in the protein production by the cell. The length of the oligonucleotide is at least 10 nucleotides and may be as long as the naturally-occurring the transcript. Preferably, the oligonucleotide is 19-25 nucleotides in length. Most preferably, the oligonucleotide is less than 75, 50 , 25 nucleotides in length. Examples of FGF18 siRNA oligonucleotide which inhibit the expression in mammalian cells include the target sequence containing SEQ ID NO: 21.

The nucleotide sequence of the siRNAs were designed using a siRNA design computer program available from the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). The computer program selects nucleotide sequences for siRNA synthesis based on the following protocol.

Selection of siRNA Target Sites:

1. Beginning with the AUG start codon of the object transcript, scan downstream for AA dinucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. Tuschl, et al. recommend against designing siRNA to the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within

75 bases) as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with the binding of the siRNA endonuclease complex.

2. Compare the potential target sites to the human genome database and eliminate from consideration any target sequences with significant homology to other coding sequences. The homology search can be performed using BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/

3. Select qualifying target sequences for synthesis. At Ambion, preferably several target sequences can be selected along the length of the gene for evaluation

The antisense oligonucleotide or siRNA of the invention inhibit the expression of the polypeptide of the invention and is thereby useful for suppressing the biological activity of the polypeptide of the invention. Also, expression-inhibitors, comprising the antisense oligonucleotide or siRNA of the invention, are useful in the point that they can inhibit the biological activity of the polypeptide of the invention. Therefore, a composition comprising the antisense oligonucleotide or siRNA of the present invention are useful in treating a CRC.

Alternatively, function of gene product of the over-expressed gene is inhibited by administering a compound that binds to or otherwise inhibits the function of the gene products. For example, the compound is an antibody which binds to the over-expressed gene product.

The present invention refers to the use of antibodies, particularly antibodies against a protein encoded by an up-regulated marker gene, or a fragment of the antibody. As used herein, the term "antibody" refers to an immunoglobulin molecule having a specific structure, that interacts (i.e., binds) only with the antigen that was used for synthesizing the antibody (i.e., the up-regulated marker gene product) or with an antigen closely related to it. Furthermore, an antibody may be a fragment of an antibody or a modified antibody, so long as it binds to the protein encoded by the marker gene. For instance, the antibody fragment may be Fab, $F(ab')_2$, Fv, or single chain Fv (scFv), in which Fv fragments from H and L chains are ligated by an appropriate linker (Huston J. S. et al. Proc. Natl. Acad. Sci. U.S.A. 85:5879-5883 (1988)). More specifically, an antibody fragment may be generated by treating an antibody with an enzyme, such as papain or pepsin. Alternatively, a gene encoding the antibody fragment may be constructed, inserted into an expression vector, and expressed in an appropriate host cell (see, for example, Co M. S. et al. J. Immunol. 152:2968-2976 (1994); Better M. and Horwitz A. H. Methods Enzymol. 178:476-496 (1989); Pluckthun A. and Skerra A. Methods Enzymol. 178:497-515 (1989); Lamoyi E. Methods Enzymol. 121:652-663 (1986); Rousseaux J. et al.

Methods Enzymol. 121:663-669 (1986); Bird R. E. and Walker B. W. Trends Biotechnol. 9:132-137 (1991)).

An antibody may be modified by conjugation with a variety of molecules, such as polyethylene glycol (PEG). The present invention provides such modified antibodies. The modified antibody can be obtained by chemically modifying an antibody. These modification methods are conventional in the field.

Alternatively, an antibody may be obtained as a chimeric antibody, between a variable region derived from a nonhuman antibody and a constant region derived from a human antibody, or as a humanized antibody, comprising the complementarity determining region (CDR) derived from a nonhuman antibody, the frame work region (FR) derived from a human antibody, and the constant region. Such antibodies can be prepared by using known technologies.

Cancer therapies directed at specific molecular alterations that occur in cancer cells have been validated through clinical development and regulatory approval of anti-cancer drugs such as trastuzumab (Herceptin) for the treatment of advanced breast cancer, imatinib methylate (Gleevec) for chronic myeloid leukemia, gefitinib (Iressa) for non-small cell lung cancer (NSCLC), and rituximab (anti-CD20 mAb) for B-cell lymphoma and mantle cell lymphoma (Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin Cancer Res. 2001 Oct;7(10):2958-70. Review.; Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001 Mar 15;344(11):783-92.; Rehwald U, Schulz H, Reiser M, Sieber M, Staak JO, Morschhauser F, Driessen C, Rudiger T, Muller-Hermelink K, Diehl V, Engert A. Treatment of relapsed CD20+ Hodgkin lymphoma with the monoclonal antibody rituximab is effective and well tolerated: results of a phase 2 trial of the German Hodgkin Lymphoma Study Group. Blood. 2003 Jan 15;101(2):420-424.; Fang G, Kim CN, Perkins CL, Ramadevi N, Winton E, Wittmann S and Bhalla KN. (2000). Blood, 96, 2246-2253.). These drugs are clinically effective and better tolerated than traditional anti-cancer agents because they target only transformed cells. Hence, such drugs not only improve survival and quality of life for cancer patients, but also validate the concept of molecularly targeted cancer therapy. Furthermore, targeted drugs can enhance the efficacy of standard chemotherapy when used in combination with it (Gianni L. (2002). Oncology, 63 Suppl 1, 47-56.; Klejman A, Rushen L, Morrione A, Slupianek A and Skorski T. (2002). Oncogene, 21, 5868-5876.). Therefore, future cancer treatments will probably involve combining

conventional drugs with target-specific agents aimed at different characteristics of tumor cells such as angiogenesis and invasiveness.

These modulatory methods are performed *ex vivo* or *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). The method involves administering a protein or combination of proteins or a nucleic acid molecule or combination of nucleic acid, molecules as therapy to counteract aberrant expression or activity of the differentially expressed genes.

Diseases and disorders that are characterized by increased (relative to a subject not suffering from the disease or disorder) levels or biological activity of the genes may be treated with therapeutics that antagonize (*i.e.*, reduce or inhibit) activity of the over-expressed gene or genes. Therapeutics that antagonize activity are administered therapeutically or prophylactically.

Therapeutics that may be utilized include, *e.g.*, (*i*) a polypeptide, or analogs, derivatives, fragments or homologs thereof of the over-expressed sequence (*ii*) antibodies to the over-expressed sequence (*iii*) nucleic acids encoding the over-expressed sequence; (*iv*) antisense nucleic acids or nucleic acids that are "dysfunctional" (*i.e.*, due to a heterologous insertion within the coding sequence of over-expressed sequence); (*v*) small interfering RNA (siRNA); or (*vi*) modulators (*i.e.*, inhibitors, agonists and antagonists that alter the interaction between an over - expressed polypeptide and its binding partner. The dysfunctional antisense molecule is utilized to "knockout" endogenous function of a polypeptide by homologous recombination (see, *e.g.*, Capecchi, *Science* 244: 1288-1292 1989) .

Increased level can be readily detected by quantifying peptide and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or peptide levels, structure and/or activity of the expressed peptides (or mRNAs of a gene whose expression is altered). Methods that are well-known within the art include, but are not limited to, immunoassays (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect expression of mRNAs (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.).

Prophylactic administration occurs prior to the manifestation of overt clinical symptoms of disease, such that a disease or disorder is prevented or, alternatively, delayed in its progression.

Therapeutic methods include contacting a cell with an agent that modulates one or more of the activities of the gene product of the differentially expressed gene. An agent that

modulates protein activity includes a nucleic acid or a protein, a naturally-occurring cognate ligand of these proteins, a peptide, a peptidomimetic, or other small molecule.

The present invention also relates to a method of treating or preventing CRC in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid of FGF18 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide or the fragment thereof. An administration of the polypeptide induce an anti-tumor immunity in a subject. To inducing anti-tumor immunity, a polypeptide encoded by a nucleic acid of FGF18 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide is administered. The polypeptide or the immunologically active fragments thereof are useful as vaccines against CRC. In some cases the proteins or fragments thereof may be administered in a form bound to the T cell recepor (TCR) or presented by an antigen presenting cell (APC), such as macrophage, dendritic cell (DC), or B-cells. Due to the strong antigen presenting ability of DC, the use of DC is most preferable among the APCs.

In the present invention, vaccine against CRC refers to a substance that has the function to induce anti-tumor immunity upon inoculation into animals. According to the present invention, polypeptides encoded by a nucleic acid of FGF18 or fragments thereof were suggested to be HLA-A24 or HLA-A*0201 restricted epitopes peptides that may induce potent and specific immune response against CRC cells expressing FGF18. Thus, the present invention also encompasses method of inducing anti-tumor immunity using the polypeptides. In general, anti-tumor immunity includes immune responses such as follows:

- induction of cytotoxic lymphocytes against tumors,
- induction of antibodies that recognize tumors, and
- induction of anti-tumor cytokine production.

Therefore, when a certain protein induces any one of these immune responses upon inoculation into an animal, the protein is decided to have anti-tumor immunity inducing effect. The induction of the anti-tumor immunity by a protein can be detected by observing in vivo or in vitro the response of the immune system in the host against the protein.

For example, a method for detecting the induction of cytotoxic T lymphocytes is well known. A foreign substance that enters the living body is presented to T cells and B cells by the action of antigen presenting cells (APCs). T cells that respond to the antigen presented by APC in antigen specific manner differentiate into cytotoxic T cells (or cytotoxic T lymphocytes; CTLs) due to stimulation by the antigen, and then proliferate (this is referred to as activation of T

cells). Therefore, CTL induction by a certain peptide can be evaluated by presenting the peptide to T cell by APC, and detecting the induction of CTL. Furthermore, APC has the effect of activating CD4+ T cells, CD8+ T cells, macrophages, eosinophils, and NK cells. Since CD4+ T cells and CD8+ T cells are also important in anti-tumor immunity, the anti-tumor immunity inducing action of the peptide can be evaluated using the activation effect of these cells as indicators.

A method for evaluating the inducing action of CTL using dendritic cells (DCs) as APC is well known in the art. DC is a representative APC having the strongest CTL inducing action among APCs. In this method, the test polypeptide is initially contacted with DC, and then this DC is contacted with T cells. Detection of T cells having cytotoxic effects against the cells of interest after the contact with DC shows that the test polypeptide has an activity of inducing the cytotoxic T cells. Activity of CTL against tumors can be detected, for example, using the lysis of ^{51}Cr-labeled tumor cells as the indicator. Alternatively, the method of evaluating the degree of tumor cell damage using ^{3}H-thymidine uptake activity or LDH (lactose dehydrogenase)-release as the indicator is also well known.

Apart from DC, peripheral blood mononuclear cells (PBMCs) may also be used as the APC. The induction of CTL is reported that the it can be enhanced by culturing PBMC in the presence of GM-CSF and IL-4. Similarly, CTL has been shown to be induced by culturing PBMC in the presence of keyhole limpet hemocyanin (KLH) and IL-7.

The test polypeptides confirmed to possess CTL inducing activity by these methods are polypeptides having DC activation effect and subsequent CTL inducing activity. Therefore, polypeptides that induce CTL against tumor cells are useful as vaccines against tumors. Furthermore, APC that acquired the ability to induce CTL against tumors by contacting with the polypeptides are useful as vaccines against tumors. Furthermore, CTL that acquired cytotoxicity due to presentation of the polypeptide antigens by APC can be also used as vaccines against tumors. Such therapeutic methods for tumors using anti-tumor immunity due to APC and CTL are referred to as cellular immunotherapy.

Generally, when using a polypeptide for cellular immunotherapy, efficiency of the CTL-induction is known to increase by combining a plurality of polypeptides having different structures and contacting them with DC. Therefore, when stimulating DC with protein fragments, it is advantageous to use a mixture of multiple types of fragments.

Alternatively, the induction of anti-tumor immunity by a polypeptide can be confirmed by observing the induction of antibody production against tumors. For example, when

antibodies against a polypeptide are induced in a laboratory animal immunized with the polypeptide, and when growth of tumor cells is suppressed by those antibodies, the polypeptide can be determined to have an ability to induce anti-tumor immunity.

Anti-tumor immunity is induced by administering the vaccine of this invention, and the induction of anti-tumor immunity enables treatment and prevention of CRC. Therapy against cancer or prevention of the onset of cancer includes any of the steps, such as inhibition of the growth of cancerous cells, involution of cancer, and suppression of occurrence of cancer. Decrease in mortality of individuals having cancer, decrease of tumor markers in the blood, alleviation of detectable symptoms accompanying cancer, and such are also included in the therapy or prevention of cancer. Such therapeutic and preventive effects are preferably statistically significant. For example, in observation, at a significance level of 5% or less, wherein the therapeutic or preventive effect of a vaccine against cell proliferative diseases is compared to a control without vaccine administration. For example, Student's t-test, the Mann-Whitney U-test, or ANOVA may be used for statistical analyses.

The above-mentioned protein having immunological activity or a vector encoding the protein may be combined with an adjuvant. An adjuvant refers to a compound that enhances the immune response against the protein when administered together (or successively) with the protein having immunological activity. Examples of adjuvants include cholera toxin, salmonella toxin, alum, and such, but are not limited thereto. Furthermore, the vaccine of this invention may be combined appropriately with a pharmaceutically acceptable carrier. Examples of such carriers are sterilized water, physiological saline, phosphate buffer, culture fluid, and such. Furthermore, the vaccine may contain as necessary, stabilizers, suspensions, preservatives, surfactants, and such. The vaccine is administered systemically or locally. Vaccine administration may be performed by single administration, or boosted by multiple administrations.

When using APC or CTL as the vaccine of this invention, tumors can be treated or prevented, for example, by the ex vivo method. More specifically, PBMCs of the subject receiving treatment or prevention are collected, the cells are contacted with the polypeptide ex vivo, and following the induction of APC or CTL, the cells may be administered to the subject. APC can be also induced by introducing a vector encoding the polypeptide into PBMCs ex vivo. APC or CTL induced in vitro can be cloned prior to administration. By cloning and growing cells having high activity of damaging target cells, cellular immunotherapy can be performed more effectively. Furthermore, APC and CTL isolated in this manner may be used for cellular

immunotherapy not only against individuals from whom the cells are derived, but also against similar types of tumors from other individuals.

Furthermore, a pharmaceutical composition for treating or preventing a cell proliferative disease, such as cancer, comprising a pharmaceutically effective amount of the polypeptide of the present invention is provided. The pharmaceutical composition may be used for raising anti tumor immunity.

Pharmaceutical compositions for inhibiting CRC

Pharmaceutical formulations include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration, or for administration by inhalation or insufflation. Preferably, administration is intravenous. The formulations are optionally packaged in discrete dosage units.

Pharmaceutical formulations suitable for oral administration include capsules, cachets or tablets, each containing a predetermined amount of the active ingredient. Formulations also include powders, granules or solutions, suspensions or emulsions. The active ingredient os optionally administered as a bolus electuary or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrant or wetting agents. A tablet may be made by compression or molding, optionally with one or more formulational ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may be coated according to methods well known in the art. Oral fluid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives. The tablets may optionally be formulated so as to provide slow or controlled release of the active ingredient therein. A package of tablets may contain one tablet to be taken on ech of the month.

Formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which

render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Alternatively, the formulations may be presented for continuous infusion. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations for rectal administration include suppositories with standard carriers such as cocoa butter or polyethylene glycol. Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges, which contain the active ingredient in a flavored base such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a base such as gelatin and glycerin or sucrose and acacia. For intra-nasal administration the compounds of the invention may be used as a liquid spray or dispersible powder or in the form of drops. Drops may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents.

For administration by inhalation the compounds are conveniently delivered from an insufflator, nebulizer, pressurized packs or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichiorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the compounds may take the form of a dry powder composition, for example a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form, in for example, capsules, cartridges, gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflators.

Other formulations include implantable devices and adhesive patches; which release a therapeutic agent.

When desired, the above described formulations, adapted to give sustained release of the active ingredient, may be employed. The pharmaceutical compositions may also contain other active ingredients such as antimicrobial agents, immunosuppressants or preservatives.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

Preferred unit dosage formulations are those containing an effective dose, as recited below, or an appropriate fraction thereof, of the active ingredient.

For each of the aforementioned conditions, the compositions, e.g., polypeptides and organic compounds are administered orally or via injection at a dose of from about 0.1 to about 250 mg/kg per day. The dose range for adult humans is generally from about 5 mg to about 17.5 g/day, preferably about 5 mg to about 10 g/day, and most preferably about 100 mg to about 3 g/day. Tablets or other unit dosage forms of presentation provided in discrete units may conveniently contain an amount which is effective at such dosage or as a multiple of the same, for instance, units containing about 5 mg to about 500 mg, usually from about 100 mg to about 500 mg.

The dose employed will depend upon a number of factors, including the age and sex of the subject, the precise disorder being treated, and its severity. Also the route of administration may vary depending upon the condition and its severity.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLE 1 MATERIALS AND METHODS

Cell lines and clinical materials.

Human colon-cancer cell lines SW480 HCT116 and DLD1, and murine fibroblast line NIH3T3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Human colon-cancer cell lines SNUC4 and SNUC5 were obtained from the Korean cell line bank (KCLB, Seoul, Korea). All of the cells were cultured as monolayers in appropriate media, as follows: Leibovitz's L-15 (Invitrogen, Carlsbad, CA) for SW480, MaCoy's 5A (Invitrogen) for HCT116, RPMI1640 (Sigma-Aldrich Corporation, St. Louis, MO) for DLD1, SNUC4 and SNUC5, and DMEM (Sigma-Aldrich) for NIH3T3; each was supplemented with 0.5% or 10% fetal bovine serum (Cansera International Inc., Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2 (HCT116, DLD1, SNUC4, SNUC5, and NIH3T3) or without CO_2 (SW480). Cancerous tissues and corresponding non-cancerous mucosae were excised from 12 patients during surgery,

after informed consent had been obtained.

Semi-quantitative RT-PCR.

Total RNA was extracted from cultured cells and clinical tissues using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Extracted RNA was treated with DNaseI (Roche Diagnostics, Mannheim, Germany) and reversely transcribed to single-stranded cDNAs using oligo(dT)$_{12-18}$ primer with Superscript II reverse transcriptase (Invitrogen). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) gene as a quantitative control. Primer sequences were 5'-ACAACAGCCTCAAGATCATCAG-3'(SEQ ID NO: 1) and 5'-GGTCCACCACTGACACGTTG-3'(SEQ ID NO: 2) for *GAPDH*, and 5'-GGACATGTGCAGGCTGGGCTA-3'(SEQ ID NO: 3) and 5'-GTAGAATTCCGTCTCCTTGCCCTT-3'(SEQ ID NO: 4) for *FGF18*. All of the reactions involved initial denaturation at 94°C for 2 min followed by 18 cycles (for *GAPDH*) or 33 cycles (for *FGF18*) at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

Northern blotting.

Human multiple-tissue blots (BD Bioscience, Palo Alto, CA) were hybridized with a ^{32}P-labeled PCR product of FGF18, which had been labeled by random-oligonucleotide priming with a Mega Label kit (Amersham Biosciences, Buckinghamshire, UK). The product was prepared by RT-PCR using primers 5'-GGACATGTGCAGGCTGGGCTA-3'(SEQ ID NO: 3) and 5'-GTGTTGGTTTCCTCATTCAAGTC-3'(SEQ ID NO: 5). Prehybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at –80 °C for 240 h.

Preparation of polyclonal antibody against FGF18.

Plasmids expressing His-tagged carboxyl-terminal FGF18 protein (codons from 167 to 207) were prepared using pET28 vector (Novagen, Madison, WI). The recombinant protein was expressed in *E. coli* BL21 codon-plus strain (Stratagene, La Jolla, CA), and purified using TALON resin (BD Bioscience) according to the supplier's protocol. The protein was inoculated into rabbits and the immune sera were purified on affinity columns according to standard methodology.

Immunohistochemistry.

Immunohistochemical staining was carried out using affinity-purified anti-FGF18 antibody against human FGF18. Frozen tissue sections were subjected to the SAB-PO peroxidase immunostaining system (Nichirei, Tokyo, Japan) according to the manufacturer's recommended method.

Effect of FGF18 on cell survival *in vitro*.

The entire coding region of human FGF18 was amplified by RT-PCR using primers 5'-CCTCAAGCTTAGCGATGTATTCA-3'(SEQ ID NO: 6) and either 5'-CGGTCTAGACTAGGCAGGGTGT-3'(SEQ ID NO: 7) or 5'-CCTCTCTCGAGGGCAGGGTGTGT-3'(SEQ ID NO: 8), and cloned into appropriate cloning sites of expression vectors pcDNA3.1(+) (Invitrogen) or pFlagCMV5 (Sigma-Aldrich). Plasmids expressing FGF18 (pcDNA-FGF18 or pFlag-FGF18) or empty vector (pcDNA or pFlagCMV5) were transfected into murine fibroblast NIH3T3 cells for a focus-formation assay. One week after transfection, the cells were fixed with 100% methanol and stained with Giemsa solution. The medium supporting NIH3T3 cells transfected with either pFlag-FGF18 or empty vector was conditioned with 0.5% FBS; growth of these cells was analyzed by MTT assay.

Construction of psiH1BX.

Transcription of the *H1RNA* gene by RNA polymerase III produces short transcripts with uridines at the 3' ends. We amplified a genomic fragment containing the promoter region of *H1RNA* by PCR, using primers 5'-TGGTAGCCAAGTGCAGGTTATA-3' (SEQ ID NO: 9), and 5'- CCAAAGGGTTTCTGCAGTTTCA-3'(SEQ ID NO: 10) and human placental DNA as a template. The product was purified and cloned into pCR2.1 plasmid vector using a TA cloning kit, according to the supplier's protocol (Invitrogen). The *Bam*HI, *Xho*I fragment containing *H1RNA* was purified and cloned into pcDNA3.1(+) between nucleotides 56 and 1257, and the fragment was amplified by PCR using primers 5'-TGCGGATCCAGAGCAGATTGTACTGAGAGT-3'(SEQ ID NO: 11) and 5'-CTCTATCTCGAGTGAGGCGGAAAGAACCA-3'(SEQ ID NO: 12). The ligated DNA became the template for PCR amplification with primers 5'-TTTAAGCTTGAAGACCATTTTTGGAAAAAAAAAAAAAAAAAAAAACA-3'(SEQ ID NO: 13) and 5'-TTTAAGCTTGAAGACATGGGAAAGAGTGGTCTCA-3' (SEQ ID NO: 14).

The product was digested with *Hin*dIII and subsequently self-ligated to produce a psiH1BX vector plasmid.

An siRNA expression vector against *FGF18* (psiH1BX-FGF18) was prepared by cloning double-stranded oligonucleotides 5'-TCCCGGTTCTGGAGAACAACTACTTCAAGAGAGTAGTTGTTCTCCAGAACC-3'(SEQ ID NO: 15) and 5'-AAAAGGTTCTGGAGAACAACTACTCTCTTGAAGTAGTTGTTCTCCAGAACC-3'(SEQ ID NO: 16) into the *Bbs*I site in the psiH1BX vector. A control plasmid, psiH1BX-EGFP, was prepared by cloning double-stranded oligonucleotides 5'-CACCGAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAAGTCGTGCTGCTTC-3'(SEQ ID NO: 17) and 5'-AAAAGAAGCAGCACGACTTCTTCTCTCTTGAAGAAGAAGTCGTGCTGCTTC-3'(SEQ ID NO: 18) into the *Bbs*I site in the psiH1BX vector.

Suppression of FGF18 expression by siRNAs.

SW480, HCT116, DLD1, and SNUC4 cells plated onto 10-cm dishes (4x 10^5 cells/dish) were transfected with FGF18 siRNA expression plasmids using FuGene6 reagent (Roche diagnostics) and maintained in media containing 10% fetal bovine serum with appropriate concentrations of Geneticin. The cells were then fixed with 100% methanol and stained with Giemsa solution. Viable cells were measured with a cell-counting kit (DOJINDO, Kumamoto, Japan). Expression of *FGF18* in the treated cells was examined by semi-quantitative RT-PCR 24 h after transfection.

MTT assay.

Cells (1×10^5) on 6-well plates were transfected with expression vector or control vector using FuGene6 (Roche diagnostics) according to the supplier's protocol. Cell viability was evaluated by MTT assay seven days after transfection. Cell-counting kit-8 (DOJINDO) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for an additional 4 h; then absorbance was measured at 490 nm, and at 630 nm as reference, with a Microplate Reader 550 (Bio-Rad Laboratories, Hercules, CA).

Reporter assay.

An initiation site (TIS) for transcription of FGF18 was determined by comparing the

human genomic sequence (GenBank accession no. AC093246) with the cDNA sequence of *FGF18* (GenBank accession no. NM_003862). To examine activity of the *FGF18* promoter we amplified by PCR four fragments, each corresponding to part of the region flanking *FGF18* on the 5' side, and cloned each product into an appropriate enzyme site of pGL3-Basic vector (Promega, Madison, WI). Plasmids expressing an activated form of ß-catenin (mut ß-catenin) and wild-type and dominant-negative forms of Tcf4 (wtTcf4 and dnTcf4) were prepared as described previously (22). One microgram of each reporter plasmid and 1 μg of each expression construct were co-transfected with 0.2 μg of pRL-TK plasmid (Promega) into SW480 cells using FuGENE6, to normalize the efficiency of transfection. Reporter assays were carried out using a dual-luciferase reporter assay system according to the supplier's recommendations (Promega).

Electrophoretic mobility-shift assay (EMSA).

The EMSA was performed as previously described (23) using nuclear extracts from SW480 cells. A double-stranded 16-nucleotide DNA probe was prepared by annealing FGF18F (5'-CGCCTTTGATGTGGGC-3'(SEQ ID NO: 19)) to FGF18R (5'-GCCCACATCAAAGGCG-3'(SEQ ID NO: 20)), and labeled with ^{32}P-ATP and T4 polynucleotide kinase.

Statistical analysis.

Statistical significance was analysed by ANOVA with Scheffe's *F* test, using commercially available software (Statview, SAS Institute, Cary, NC).

EXAMPLE 2. RESULTS

Up-regulation of FGF18 in CRC.

Previously we had analyzed expression profiles of nine adenomas and 11 adenocarcinomas of the colon by means of a cDNA microarray representing 23,040 genes (24). Among the genes whose expression levels were commonly up-regulated in cancer cells, a spot corresponding to *FGF18* showed high tumor/normal intensity ratios in the majority of the cases examined. The putative full-length cDNA consisted of 1546 nucleotides, with an open reading frame of 624 nucleotides (SEQ ID NO: 22) encoding a 207-amino-acid protein (SEQ ID NO: 23) (GenBank Accession number: AFO75292). Subsequent semi-quantitative RT-PCR corroborated enhanced expression of this gene in 10 of 12 additional colon-cancer tissues examined (Fig. 1A). To examine expression of *FGF18* in normal adult human tissues, we carried out northern-blot analysis and identified a transcript of approximately 1.8 kb that was

abundantly expressed in the heart but not in any of 28 other tissues examined (data not shown).

Accumulation of FGF18 in tumor cells.

To analyze the function of FGF18, we prepared an anti-FGF18 antibody that would recognize endogenous FGF18 protein in cells, and investigated expression of this protein in four colorectal-cancer tissues by immunohistochemical staining. In all four cases, FGF18 was stained in the cytoplasm of cancerous cells (Fig. 1B); staining in the cytoplasm of non-cancerous epithelial cells from corresponding mucosae was significantly weaker and localized mainly at the bottom of crypts.

Assay of the FGF18 promoter in colon-cancer cells.

Since transactivation of the ß-catenin/Tcf4 complex is a relatively common feature of colon-cancer cells, we tested whether this complex regulates expression of *FGF18* by infecting SW480 cells with adenovirus expressing a dominant negative form of Tcf4 (dnTcf4), or with a control gene (*LacZ*). Expression of *FGF18* was significantly decreased in response to dnTcf4 compared to the control, suggesting that Tcf4-mediated transcriptional activity was correlated with expression of *FGF18* (data not shown). Hence, we searched for consensus Tcf4-binding motifs, 5'-CTTTGWW-3' or 5'-WWCAAAG-3', within a two-kb genomic fragment of the 5' region flanking *FGF18*, and identified three possible candidate sites; i.e., between -1631 and -1625 (TBM1), -1348 and -1342 (TBM2), and -190 and -184 (TBM3; Fig. 2A). To examine which of these binding sites might be responsible for the promoter activity of *FGF18*, we cloned fragments of various lengths from its 5' flanking region upstream of the luciferase gene, and performed a reporter assay using SW480 cells (Fig. 2B). pGL3-P1 (containing nucleotides between –1644 and +26), pGL3-P2 (–1354 and +26), and pGL3-P3 (–195 and +26) revealed approximately 5-fold increases in luciferase activity compared with pGL3-P4 (–181 and +26), suggesting that the region between –195 and –182 was responsible for the transcriptional activity. To further clarify the role of TBM-3, we assayed the luciferase activity after introducing a 2-base mutation (CTTTGAT (SEQ ID NO: 24) to CTTTGGC) at the TBM3 site (pGL3-P3mt). As expected, mutation at the TBM3 site (P3mt) reduced luciferase activity by more than 75%. These results suggested that TBM3 indeed contained the promoter sequence for *FGF18*.

Association between the putative Tcf4 binding site and the ß-catenin/Tcf complex.

To examine whether the ß-catenin/Tcf4 complex associates with the TBM-3 site in the

promoter region of *FGF18*, we carried out an electrophoretic mobility-shift assay (EMSA) using oligonucleotides corresponding to the TBM-3 sequence. A single band was shifted by addition of anti-ß-catenin antibody, but not by an unrelated (control) antibody. This binding was abrogated by addition of wild-type unlabeled oligonucleotide, but not by mutant unlabeled oligonucleotides, indicating direct interaction between the binding sequence and the ß-catenin/Tcf4 complex (Fig. 3).

Growth advantage conferred by over-expression of FGF18 in NIH3T3 cells.

Since over-expression of *FGF18* was known to promote growth of fibroblasts and osteoblasts, we hypothesized that FGF18 could render oncogenic effects in an autocrine manner. In line with that hypothesis, our immunoblotting experiments detected Flag-tagged FGF18 protein in the culture media of murine fibroblast cells transfected with pFlagCMV-FGF18 (Fig. 4A). As expected, the NIH3T3 cells transfected with pFlagCMV-FGF18 proliferated at a significantly higher rate in conditioned media than cells transfected with mock vector (Fig. 4B).

Effect of FGF18 siRNA on growth of cancer cells.

To evaluate the potentially oncogenic role of FGF18, we prepared plasmids expressing siRNA of FGF18 and transfected them into five lines of colorectal-carcinoma cells expressing abundant amounts of FGF18. Among the constructed plasmids, psiH1BX-FGF18 significantly reduced expression of *FGF18* compared with a control plasmid (psiH1BX-EGFP), and markedly decreased the number of viable cells compared with psiH1BX-EGFP (Fig. 4C,D,E).

EXAMPLE3. DISCUSSION

We have demonstrated here that *FGF18* is frequently up-regulated in colorectal carcinomas, as a direct target of the ß-catenin/Tcf4 complex. Since FGF18 is a secreted protein, it might serve as a novel marker for early detection of colorectal tumors. Moreover, because in our experiments FGF18 protein promoted growth of NIH3T3 cells in an autocrine manner, and its down-regulation suppressed growth or survival of colon-cancer cells, FGF18 may also represent a promising molecular target for novel anticancer drugs.

FGF18 was first identified on the basis of its amino-acid similarities to *FGF8* and *FGF17* (60% and 58% identity, respectively) (18). Like other FGFs, it has an important role in limb development, probably through modulation of osteoblasts, chondrocytes, and osteoclasts (12, 25), and in organogenesis of the midbrain (26). In mice, expression of FGF18 has been

observed in the developing lung, surrounding developing bones, and cerebral cortex of the developing brain during embryonic stage E15.5 (18). Intra-peritoneal injection of recombinant FGF18 protein induced significant gains in the weights of liver and intestine in mice (18). Additionally FGF18 stimulated growth of NIH3T3 cells in a heparan sulfate-dependent manner (18). These observations agree well with our conclusion that elevated expression of FGF18 stimulates growth and/or prevents death of epithelial and mesenchymal cells in an autocrine manner.

FGFs function by binding with FGF receptors (FGFRs); five FGFR genes and their splicing variants have been identified so far. A BIAcore assay demonstrated that FGFR-3c and FGFR-2c, but not FGFR1c, have affinity for FGF18 (20). Those data suggest that FGF18 exerts its growth-promoting effect by interacting with some receptors. However, the phenotypes of FGF18-deficient mice, which show delayed ossification and decreased expression of osteogenic markers, do not conform completely with the phenotype of mice lacking FGFR-3 (27). Therefore, antagonizing FGFR-3c and/or other FGFRs, such as FGFR-2c, might be an effective strategy for suppressing FGF18-mediated cell growth.

Our experiments also revealed that FGF18 is the second member of the FGF family to be proven as a direct target of Tcf/LEF. Previously FGF4 was reported to be a direct target of LEF1; recombinant FGF4 fully rescued the $Lef^{-/-}$ phenotype for tooth development in mice (13). Wnt signals also control FGF-dependent limb initiation *via* FGF8 and FGF10 (12, 25). Since FGF18 is expressed in the right side of Hensen's node before expression of FGF8 occurs, and expression of FGF18 also precedes FGF8 in the isthmus in the developing brain, Wnt signals may recruit FGF18 as an initial mediator of organogenesis of the limb and brain. Like FGF4, which has transforming activity (17), FGF18 probably can become oncogenic when inappropriately over-expressed.

Immunohistochemical staining of FGF18 in human colon-cancer tissues and corresponding non-cancerous mucosae showed a pattern similar to that of ß-catenin and other ß-catenin/Tcf downstream proteins such as ENC1, CD44, and EPHB2 (28). This evidence supports a view that activated ß-catenin/Tcf4 complexes in colonic tumors have switched on the proliferative signals that are normally restricted to progenitor cells located in the lower third of colonic crypts. Therefore, FGF18 expressed in non-tumorous crypts may play some role in maintenance of progenitor cells, which are absent in Tcf4-knockout mice. However, since in published experiments FGF18-knockout mice did not reveal any abnormalities in intestinal structure, other factors may redundantly affect the development of mucosa (27). Furthermore,

FGF18 could exert different functions in other tissues, specifically bone and brain. Further studies on the function of genes downstream of the ß-catenin/Tcf4 transcription complex should help to clarify which factors are required for maintenance of progenitor cells in colonic epithelium.

In conclusion, our data have underscored the importance of elevated expression of *FGF18* in colorectal tumorigenesis. Since FGF18 functions as growth factor by binding its receptor(s) at least, but not solely, in an autocrine manner, our data clearly indicate that FGF18 should be a good candidate as a tumor marker as well as a molecular target for the development of reagents, such as specific neutralizing antibodies or antagonists against the receptor(s), for treatment of patients with colorectal tumors.

Industrial Applicability

The previous gene-expression analysis of genome-wide cDNA microarray has identified specific up-regulated gene *FGF18*. The present invention revealed *FGF18* serves as target for cancer prevention and therapy. Based on the expression of FGF18, the present invention provides a molecular diagnostic marker for identifying or detecting CRC.

The methods described herein are also useful in the identification of additional molecular targets for prevention, diagnosis and treatment of CRC. The data reported herein add to a comprehensive understanding of CRC, facilitate development of novel diagnostic strategies, and provide clues for identification of molecular targets for therapeutic drugs and preventative agents. Such information contributes to a more profound understanding of colorectal tumorigenesis, and provide indicators for developing novel strategies for diagnosis, treatment, and ultimately prevention of CRC.

All patents, patent applications, and publications cited herein are incorporated by reference in their entirety. Furthermore, while the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

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28. van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Battle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. The ß-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell, *111:* 241-250, 2002.

What is claimed is:

1. A method of diagnosing CRC or a predisposition to developing CRC in a subject, comprising determining a level of expression of FGF18 in a patient derived biological sample, wherein an increase of said level compared to a normal control level of said gene indicates that said subject suffers from or is at risk of developing CRC.
2. The method of claim 1, wherein said increase is at least 10% greater than said normal control level.
3. The method of claim 1, wherein the expression level is determined by any one method select from group consisting of:
(a) detecting the mRNA of FGF18,
(b) detecting the protein encoded by FGF18, and
(c) detecting the biological activity of the protein encoded by FGF18,
4. The method of claim 1, wherein said level of expression is determined by detecting hybridization of FGF18 probe to a gene transcript of said patient-derived biological sample.
5. The method of claim 4, wherein said hybridization step is carried out on a DNA array.
6. The method of claim 1, wherein said biological sample comprises an epithelial cell.
7. The method of claim 1, wherein said biological sample comprises CRC cell.
8. The method of claim 4, wherein said biological sample comprises an epithelial cell from a CRC.
9. A method of screening for a compound for treating or preventing CRC, said method comprising the steps of:
a) contacting a test compound with a polypeptide encoded by a nucleic acid of FGF18;
b) detecting the binding activity between the polypeptide and the test compound; and
c) selecting a compound that binds to the polypeptide.
10. A method of screening for a compound for treating or preventing CRC , said method comprising the steps of:
a) contacting a candidate compound with a cell expressing FGF18, and
b) selecting a compound that reduces the expression level of FGF18.
11. The method of claim 10, wherein said cell comprises a colorectal cancer cell.
12. A method of screening for a compound for treating or preventing CRC, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by a nucleic acid of FGF18;

b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by a nucleic acid of FGF18 in comparison with the biological activity detected in the absence of the test compound.

13. The method of claim 12, wherein the biological activity of the polypeptide is cell proliferative activity.

14. A method of screening for compound for treating or preventing CRC, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of FGF18 and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene, as compared to a control.

15. The method of claim13, wherein the transcriptional regulatory region comprising the ß-catenin/Tcf4 binding motief in the transcriptional regulatory region of FGF18.

16. The method of claim 14, wherein said binding motief consisting of nucleotide sequence set forth in SEQ ID NO: 24.

17. A method of screening for compound for treating or preventing CRC, said method comprising the steps of:

a) contacting a DNA comprising the ß-catenin/Tcf4 binding motief in the transcriptional regulatory region of FGF18 with ß-catenin/Tcf4 complex in the presence or absence of candidate compound;

b) detecting the binding of the DNA and the ß-catenin/Tcf4 complex; and

c) selecting a compound that inhibits the binding of the ß-catenin/Tcf4 complex with the DNA, , as compared to a control.

18. The method of claim 16, wherein said binding motief consisting of SEQ ID NO: 24.

19. A kit comprising a detection reagent which binds to nucleic acid sequence or polypeptide of FGF18.

20. A method of treating or preventing CRC in a subject comprising administering to said subject an antisense composition, said composition comprising a nucleotide sequence

complementary to a coding sequence of FGF18.

21. A method of treating or preventing CRC in a subject comprising administering to said subject a siRNA composition, wherein said composition reduces the expression of a nucleic acid sequence of FGF18.

22. The method of claim 21, wherein the siRNA comprises a sense strand comprising the nucleotide sequence of SEQ ID NO: 21.

23. A method for treating or preventing CRC in a subject comprising the step of administering to said subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by nucleic acid of FGF18.

24. A method of treating or preventing CRC in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid of FGF18 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide.

25. A method for treating or preventing CRC in a subject, said method comprising the step of administering a compound that is obtained by the method according to any one of claims 9-17.

26. A composition for treating or preventing CRC, said composition comprising a pharmaceutically effective amount of an antisense polynucleotide or small interfering RNA against a polynucleotide of FGF18.

27. The composition of claim 26, wherein the siRNA comprises a sense strand comprising the nucleotide sequence of SEQ ID NO: 21.

28. A composition for treating or preventing CRC, said composition comprising a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by nucleic acid of FGF18.

29. A composition for treating or preventing CRC, said composition comprising a pharmaceutically effective amount of the compound selected by the method of any one of claims 9-17 as an active ingredient, and a pharmaceutically acceptable carrier.

ABSTRACT

Objective methods for detecting and diagnosing colorectal cancer (CRC) are described herein. In one embodiment, the diagnostic method involves the determining a expression level of FGF18 that discriminate between CRC and nomal cell. The present invention further provides methods of screening for therapeutic agents useful in the treatment of CRC, methods of treating CRC and method of vaccinating a subject against CRC.

Fig. 1

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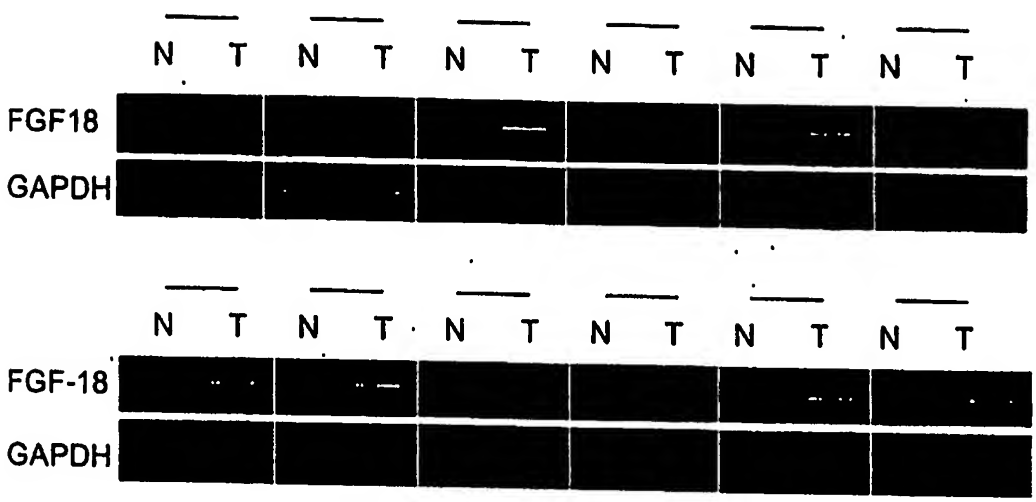

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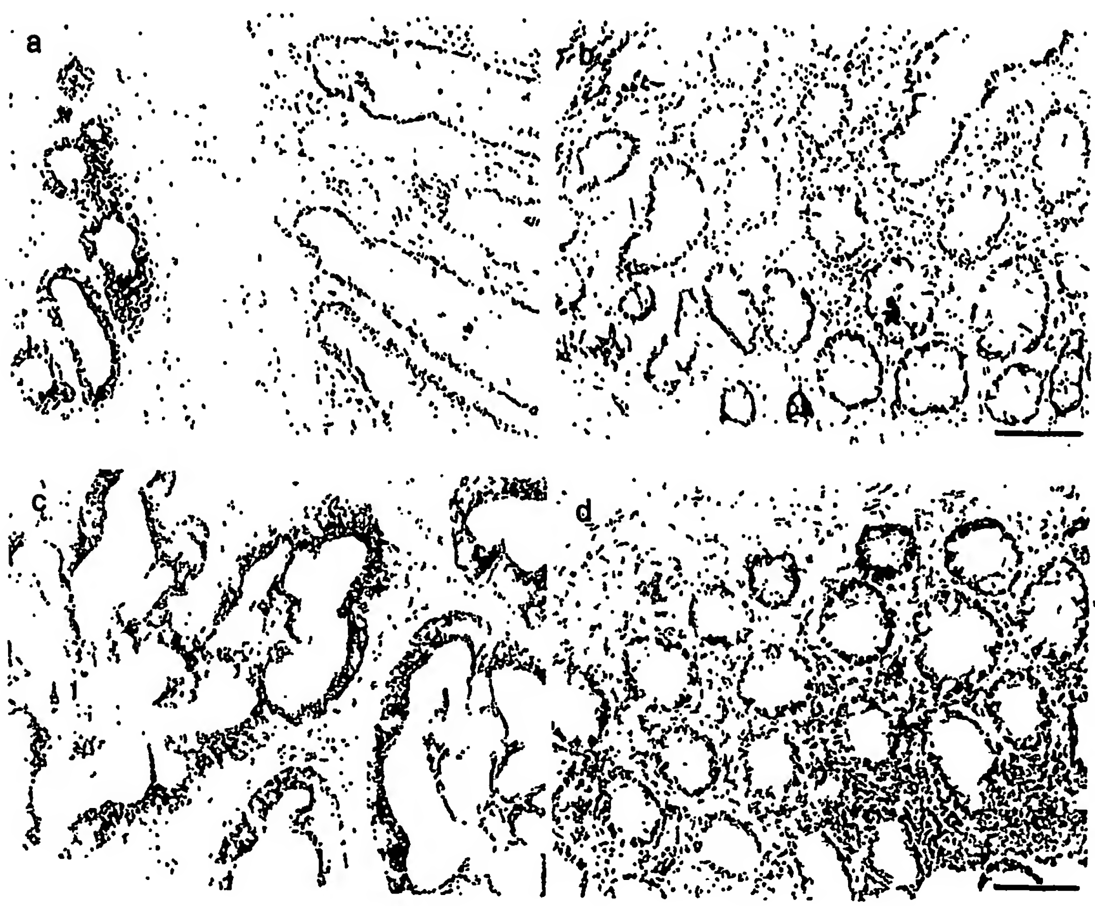

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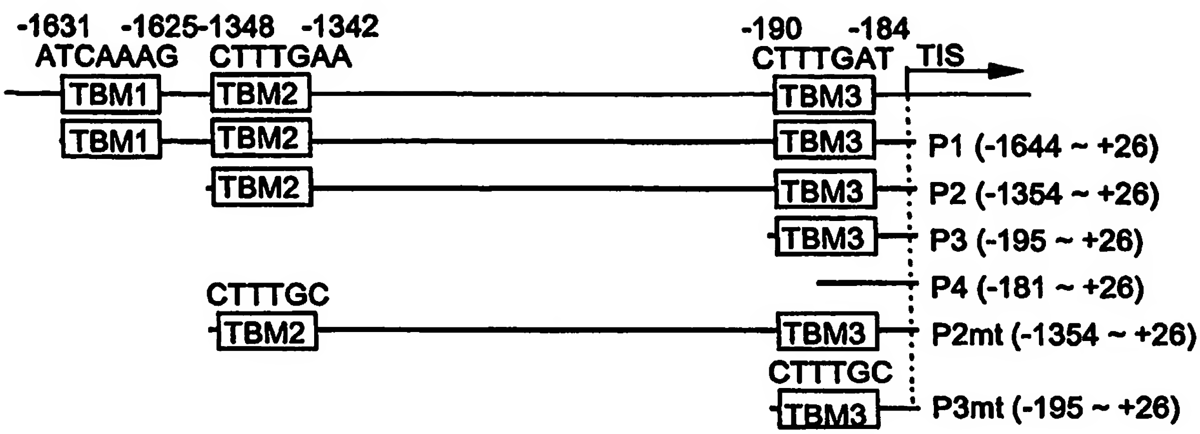
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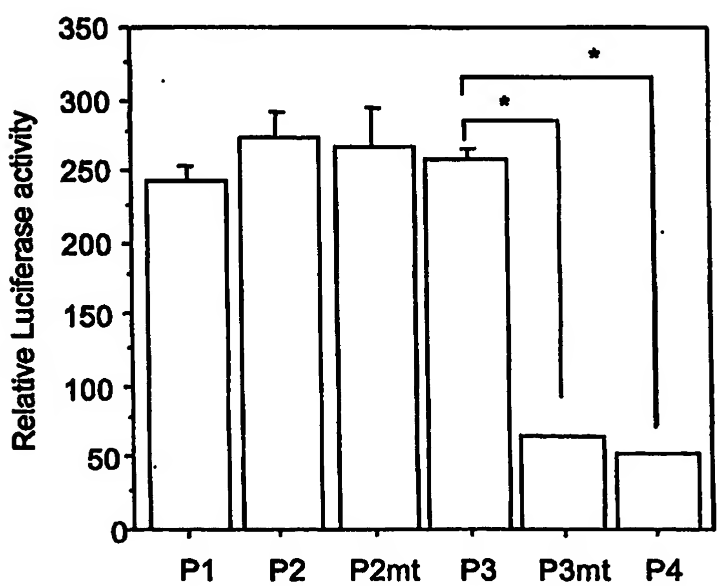
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Fig. 3

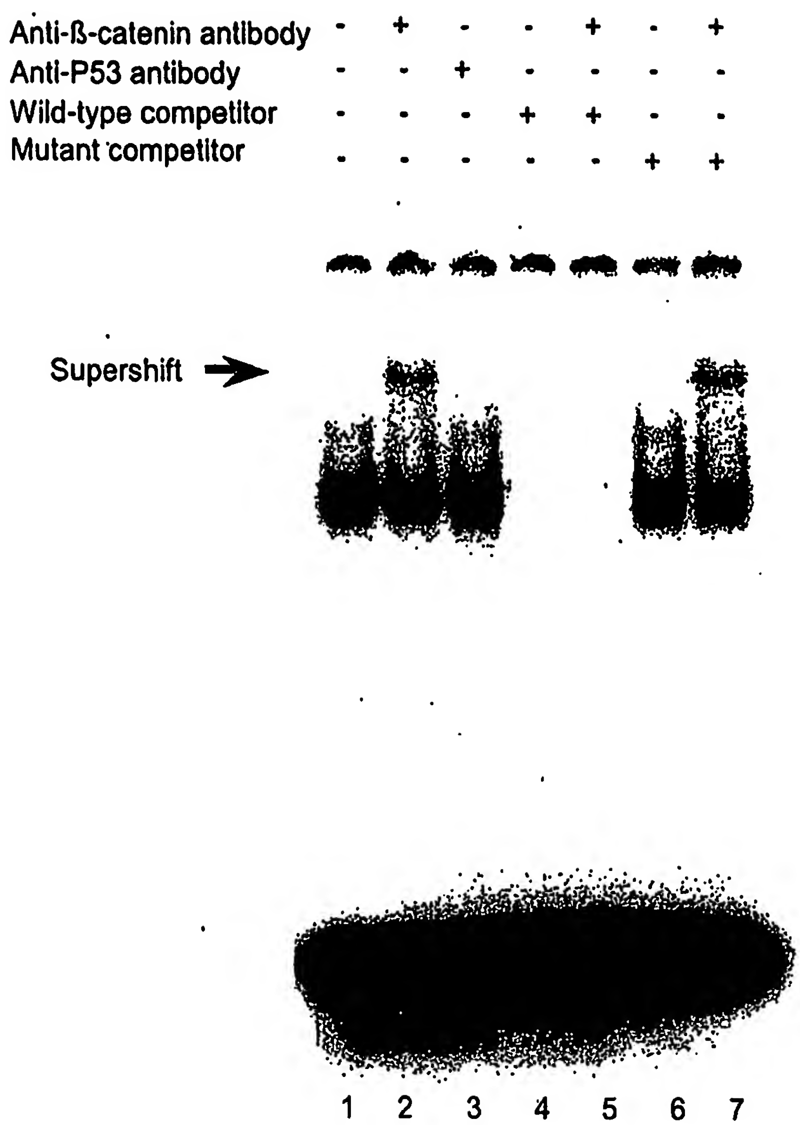
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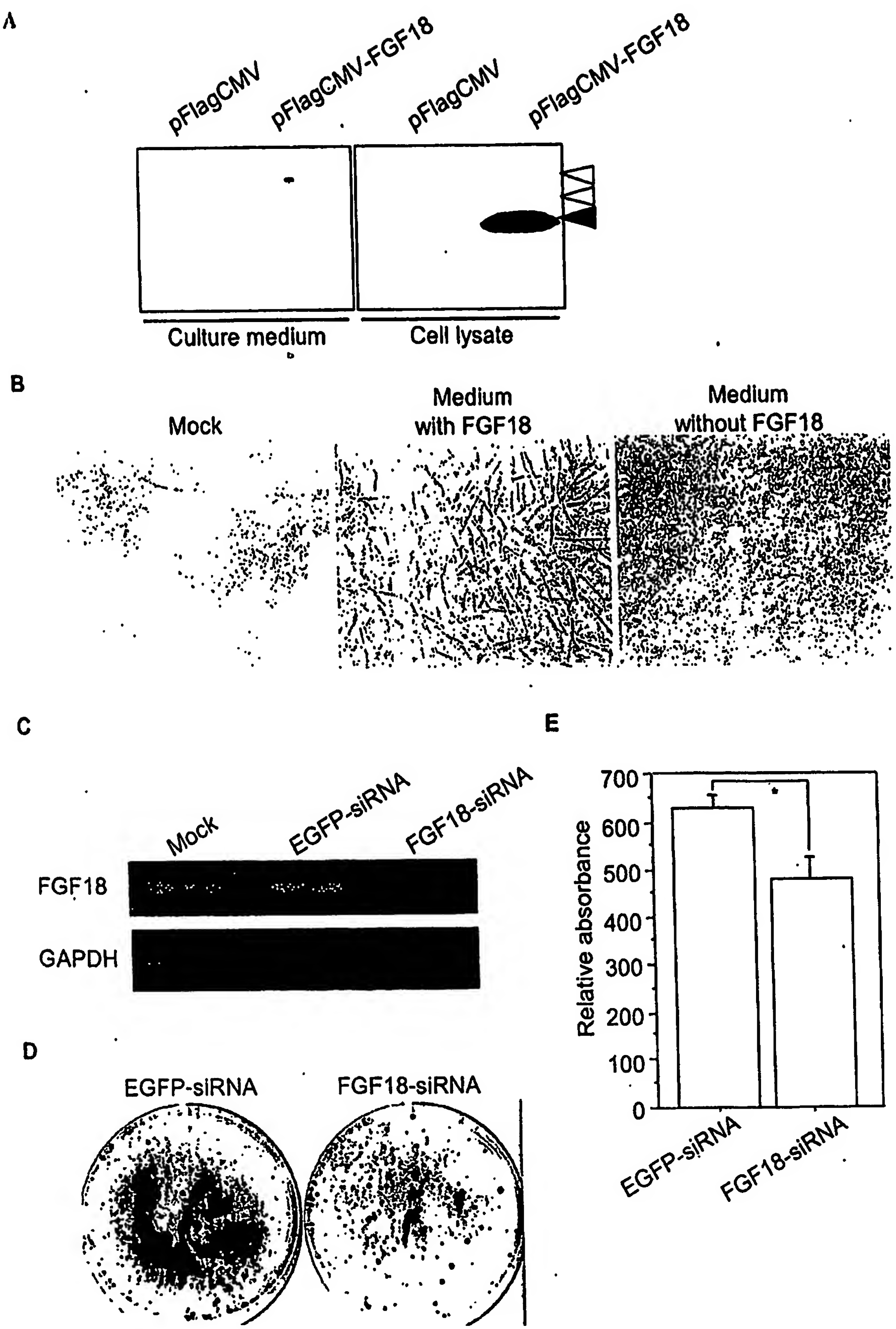
Fig. 1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D
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